

Figure 1A

DNA and Predicted Amino Acid Sequences
of the "Dimeric" Anti-CD20 Light Chain (Version 1)

	Start		[Murine Natural Leader]
	ATG GAT TTT CAG GTG CAG ATT ATC AGC TTC CTG CTA ATC AGT GCT TCA GTC ATA		
	Met Asp Phe Gln Val Gln Ile Ile Ser Phe Leu Leu Ile Ser Ala Ser Val Ile		
	ATG TCC AGA GGA CAA ATT GTT CTC TCC CAG TCT CCA GCA ATC CTG TCT GCA TCT		
	Met Ser Arg Gly Gln Ile Val Leu Ser Gln Ser Pro Ala Ile Leu Ser Ala Ser		
	CCA GGG GAG AAG GTC ACA ATG ACT TGC AGG GCC AGC TCA AGT GTA AGT TAC ATC		
15	Pro Gly Glu Lys Val Thr Met Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Ile		
			[Murine Anti-Human CD20 Light Chain Variable]
	CAC TGG TTC CAG CAG AAG CCA GGA TCC TCC CCC AAA CGC TGG ATT TAT GCC ACA		
33	His Trp Phe Gln Gln Lys Pro Gly Ser Ser Pro Lys Arg Trp Ile Tyr Ala Thr		
	TCC AAC CTG GCT TCT GGA GTC CCT GTT CGC TTC AGT GGC AGT GGG TCT GGG ACT		
51	Ser Asn Leu Ala Ser Gly Val Pro Val Arg Phe Ser Gly Ser Gly Ser Gly Thr		
	TCT TAC TCT CTC ACA ATC AGC AGA GTG GAG GCT GAA GAT GCT GCC ACT TAT TAC		
69	Ser Tyr Ser Leu Thr Ile Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr		
	TGC CAG CAG TGG ACT AGT AAC CCA CCC ACG TTC GGA GGG GGG GCC AAG CTG GAA		
87	Cys Gln Gln Trp Thr Ser Asn Pro Pro Thr Phe Gly Gly Gly Ala Lys Leu Glu		
	ATC AAA CGT ACG GTG GCT GCA CCA TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG		
105	Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu		
	CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC		
123	Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro		
			[Human Kappa Light Chain Constant]
	AGA GAG GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC		
141	Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser		

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      CAG GAG AGT GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
159 Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser

      ACC CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC TGC GAA
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
177 Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu

      GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
195 Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu

      Stop
      TGT TGA
      --- ---
213 Cys ***

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Amino acid residue numbering is sequential beginning with the amino terminus of the final protein (leader peptide removed).

Figure 2A

DNA and Predicted Amino Acid Sequences
of the "Dimeric" Anti-CD20 Heavy Chain (Version 1)

	Start		[Synthetic Leader]	
	ATG	GGT	TGG AGC CTC ATC TTG CTC TTC CTT GTC GCT GTT GCT ACG CGT GTC CTG	
	Met	Gly	Trp Ser Leu Ile Leu Leu Phe Leu Val Ala Val Ala Thr Arg Val Leu	
	TCC	CAG	GTA CAA CTG CAG CAG CCT GGG GCT GAG CTG GTG AAG CCT GGG GCC TCA	
	Ser	Gln	Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala Ser	
	GTG	AAG	ATG TCC TGC AAG GCT TCT GGC TAC ACA TTT ACC AGT TAC AAT ATG CAC	
15	Val	Lys	Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn Met His	
	TGG	GTA	AAA CAG ACA CCT GGT CGG GGC CTG GAA TGG ATT GGA GCT ATT TAT CCC	
33	Trp	Val	Lys Gln Thr Pro Gly Arg Gly Leu Glu Trp Ile Gly Ala Ile Tyr Pro	
			[Murine Anti-Human CD20 Heavy Chain Variable]	
	GGA	AAT	GGT GAT ACT TCC TAC AAT CAG AAG TTC AAA GGC AAG GCC ACA TTG ACT	
51	Gly	Asn	Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr	
	GCA	GAC	AAA TCC TCC AGC ACA GCC TAC ATG CAG CTC AGC AGC CTG ACA TCT GAG	
69	Ala	Asp	Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu	
	GAC	TCT	GCG GTC TAT TAC TGT GCA AGA TCG ACT TAC TAC GGC GGT GAC TGG TAC	
87	Asp	Ser	Ala Val Tyr Tyr Cys Ala Arg Ser Thr Tyr Tyr Gly Gly Asp Trp Tyr	
	TTC	AAT	GTC TGG GGC GCA GGG ACC ACG GTC ACC GTC TCT GCA	GCT AGC ACC AAG
105	Phe	Asn	Val Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ala	Ala Ser Thr Lys
	GGC	CCA	TCG GTC TTC CCC CTG GCA CCC TCC TCC AAG AGC ACC TCT GGG GGC ACA	
123	Gly	Pro	Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr	
	GCG	GCC	CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG	
141	Ala	Ala	Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser	

Figure 2B

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      TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG
      ---
159 Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln

      TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC AGC TTG
      ---
177 Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu

      GGC ACC CAG ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC AAC ACC AAG GTG
      ---
195 Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val

      GAC AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC
      ---
213 Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys

      CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC
      ---
231 Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro

      [Human Gamma 1 Heavy Chain Constant]
      AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC
      ---
249 Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp

      GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG
      ---
267 Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu

      GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT
      ---
285 Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg

      GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC
      ---
303 Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr

      AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC
      ---
321 Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser

      AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG
      ---
339 Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg

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Figure 2C

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      GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
357 Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr

      CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
375 Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr

      AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
393 Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys

      CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
411 Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val

      ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TGT CCG
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
429 Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Cys Pro

      Stop
      GGT AAA TGA 3'
      --- --- ---
447 Gly Lys ***

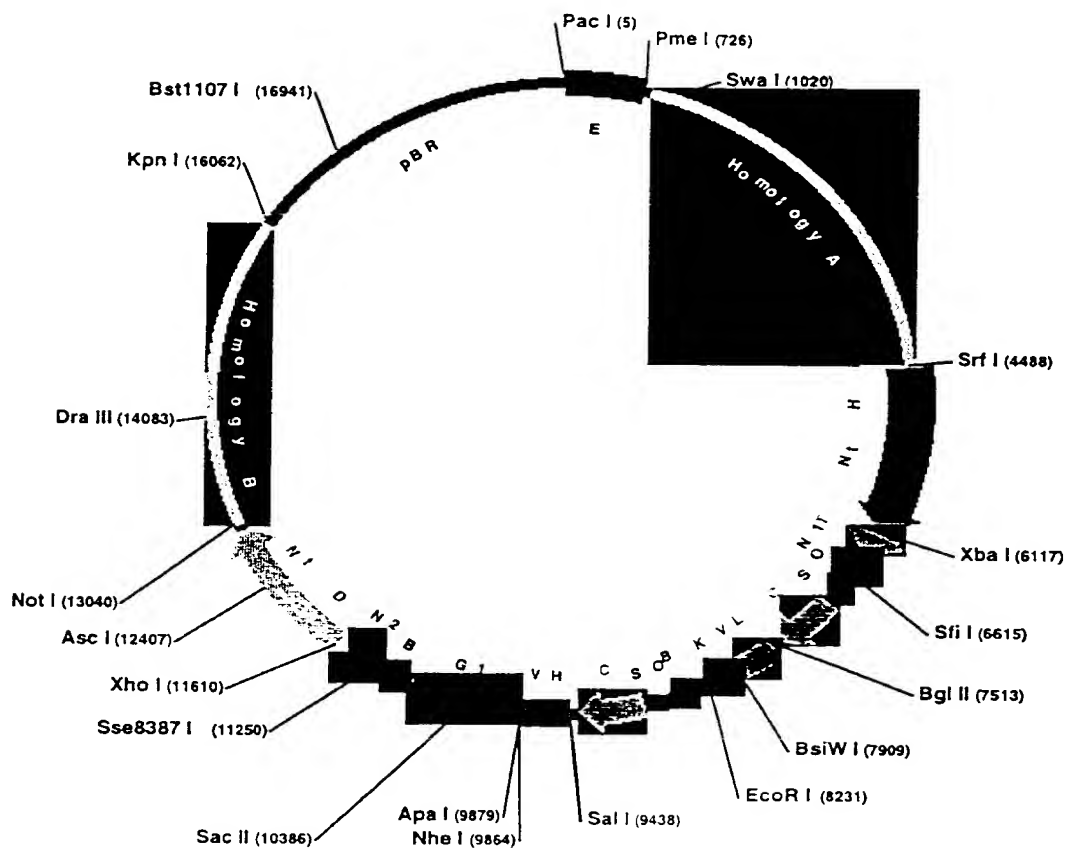
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The bold dot indicates position of the C to G transversion mutation. The normal TCT codon encoding a serine has been changed to the TGT codon encoding a cysteine amino acid.

Amino acid residue numbering is sequential beginning with the amino terminus of the final protein (leader peptide removed).

Figure 3: Schematic map of expression construct used to transfect CHO 15C9 cell line. Plasmid was linearized by restriction endonuclease digestion with Kpn I and Pac I prior to electroporation.

Dimeric Anti-CD20 in TOM-KG1(V)



Nt D = Inactive Dihydrofolate reductase
 E = CMV and SV40 enhancers
 Nt H = Inactive Salmonella Histidinol Dehydrogenase
 T = Herpes Simplex thymidine kinase promoter and polyoma enhancer
 C = Cytomegalovirus promoter/enhancer
 polyadenylation
 N1 = Neomycin phosphotransferase exon 1 M2 = Neomycin phosphotransferase exon 2
 K = Human kappa constant G1 = Dimeric Human Gamma 1 constant
 VL = Variable light chain anti-CD20 and leader
 VH = Variable heavy chain anti-CD20 and leader

SO = SV40 Origin of replication

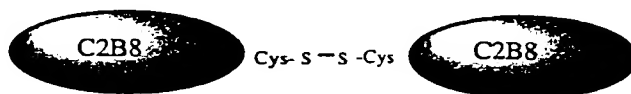
B = Bovine growth hormone

Map by Barney Barnett Constructed by Tri Huynh
 Noncutters = Afl II, Avr II, Hind III, Kpn I, Kpn II, Pml I

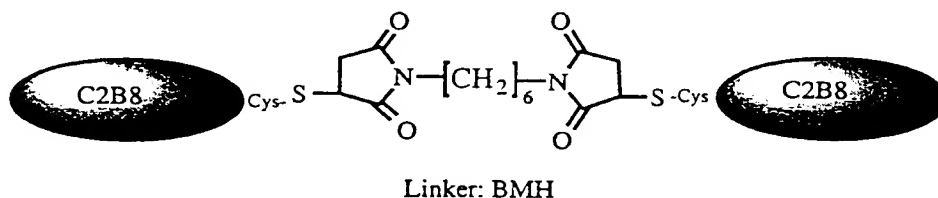
19,056 bp

Fig. 4:
Structures of C2B8 (α CD20) Homodimer and
C2B8/p5E8 Heterodimer (α CD20/ α CD23)

1. C2B8 (-S-S-) C2B8 Homodimer {Disulfide linked}



2. C2B8 (-S-) C2B8 Homodimer {Thioether linked}



3. C2B8 (-S-) p5E8 Heterodimer {Thioether linked}

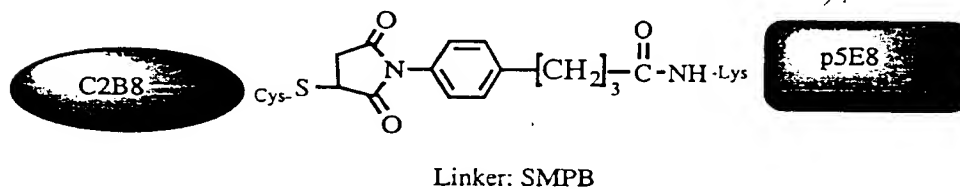


Fig. 5
SDS/PAGE Non-Reducing Gel Comparing C2B8 (-s-s-) Homodimers and C2B8/p5E8 (-s-) Heterodimers to Starting Material

Mab protein was analyzed using a 4%-20% SDS/Tris-Glycine gel under non-reducing conditions. Except for lane 2, each well received 1 ug/ml Mab protein. Individual protein bands were visualized with XXXblue stain.

MW : MW Markers

Lane 1: C2B8/SH Tissue Culture

Lane 2: C2B8/SH, Lot #: 2058-29
pA purified, (2 ug/ml)

Lane 3: C2B8/SH, Lot #: 2058-29
pA purified

Lane 4: C2B8 (-s-s-) Homodimer, Lot # 1966-76c
before HPLC purification

Lane 6: C2B8/p5E8 (-s-) Heterodimer Lot#:1977-76a
300kDa HPLC Fraction

Lane 7: C2B8, clinical Lot #:0113

Lane 8: p5E8 monomer, clone H24-31

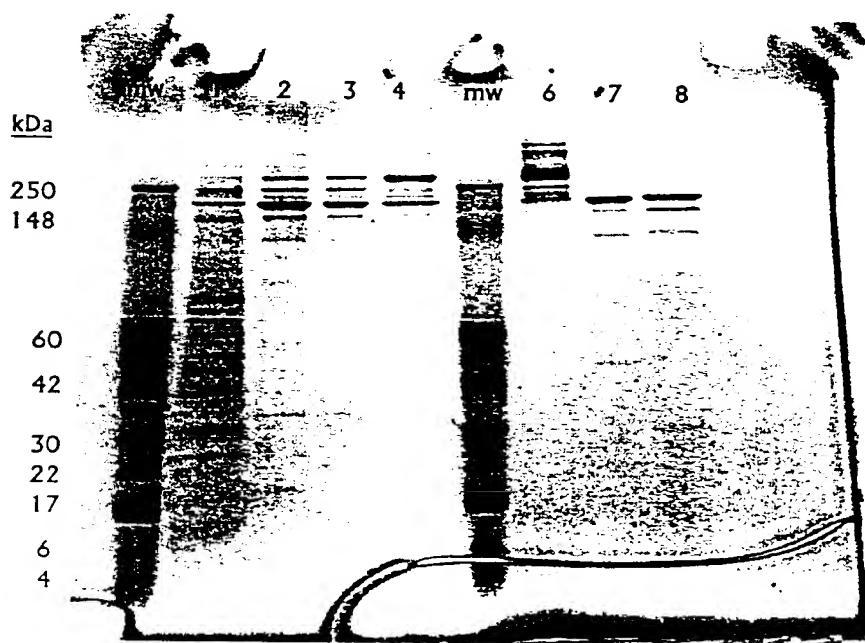


Fig. 6 **SDS/PAGE Reducing Gel Comparing C2B8 (-s-s- and -s-) Homodimers and C2B8/p5E8 (-s-) Heterodimers to Starting Material**

Mab protein was analyzed using a 4%-20% SDS/Tris-Glycine gel under reducing conditions. Mab protein was reduced using 2-mercaptoethanol and heat (90°C, 10 min) before SDS/PAGE. Individual protein bands were visualized with Comassie blue stain.

Lanes 1: MW Markers
 Lane 2: p5E8 (clone H24-31),
 pA purified
 Lane 3: C2B8/SH, Lot #: 2058-29
 pA purified,
 Lane 4: C2B8/SH, Lot #: 2058-29
 Reduced DTT

Lane 5: C2B8 (-s-s-) Homodimer, Lot # 1966-76c
 300kDa HPLC Fraction
 Lane 6: C2B8/p5E8 (-s-) Heterodimer Lot#:1977-76a
 300kDa HPLC Fraction
 Lane 7: C2B8 (-s-) Homodimer Lot #: 1966-76b
 300kDa HPLC Fraction

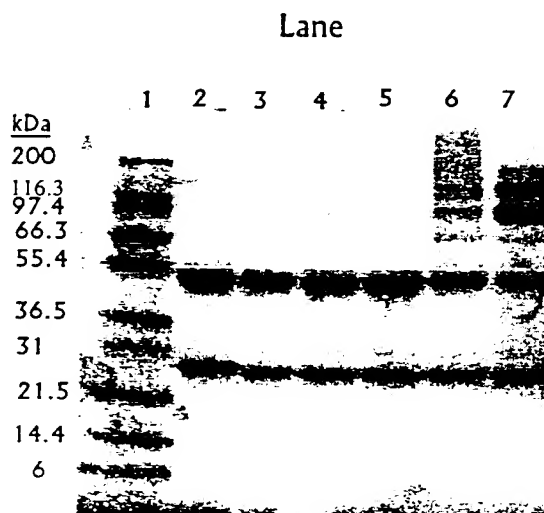


Fig.7:
HPLC Analysis of C2B8 Homodimers

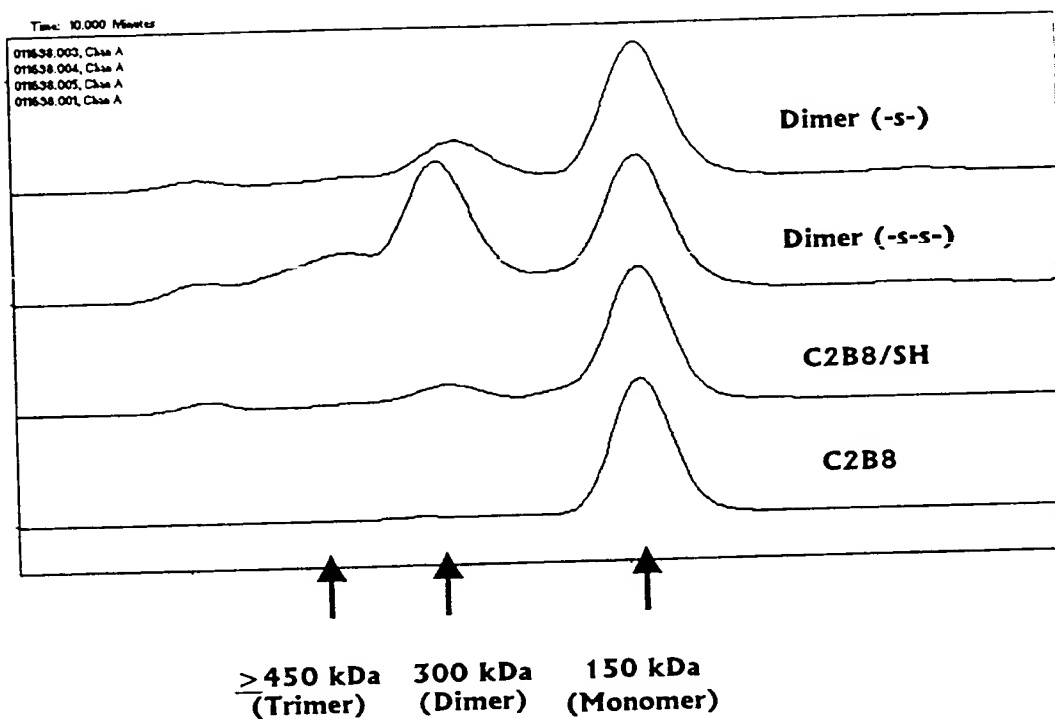


Table 1

Antibody	Percent		
	Monomer	Dimer	Aggregate
C2B8	97.2	2.8	
C2B8/SH	77.9	17.5	4.6
Dimer (-s-s-)	40.9	39.4	14.8
Dimer (-s-)	66.2	27.9	3.6

Fig 8:
HPLC Analysis of C2B8/p5E8 Heterodimers
(α CD20/ α CD23 Dimer)

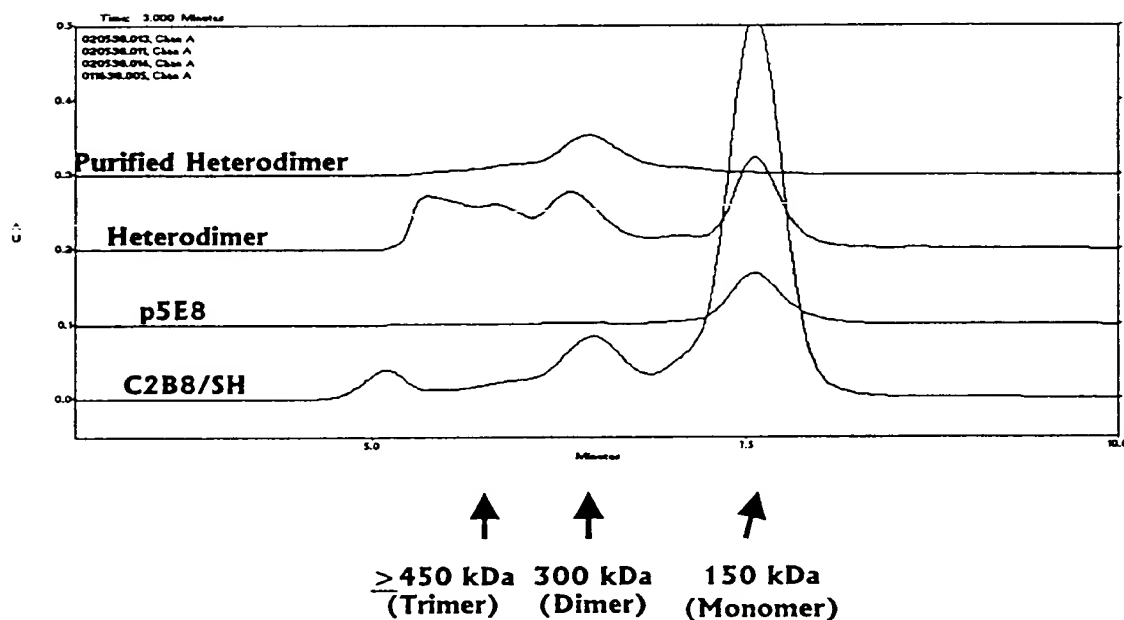


Table 2

Antibody	Percent		
	Monomer	Dimer	Aggregate
C2B8/SH	82.5	12.5	5
p5E8	99.8	0.2	
Heterodimer	34.5	26	39.5
purified Dimer	3.5	96.5	

Fig. 9
Binding of C2B8 (s-s-) Homodimer to CD20 Positive
Cell Lines: SKW and SB

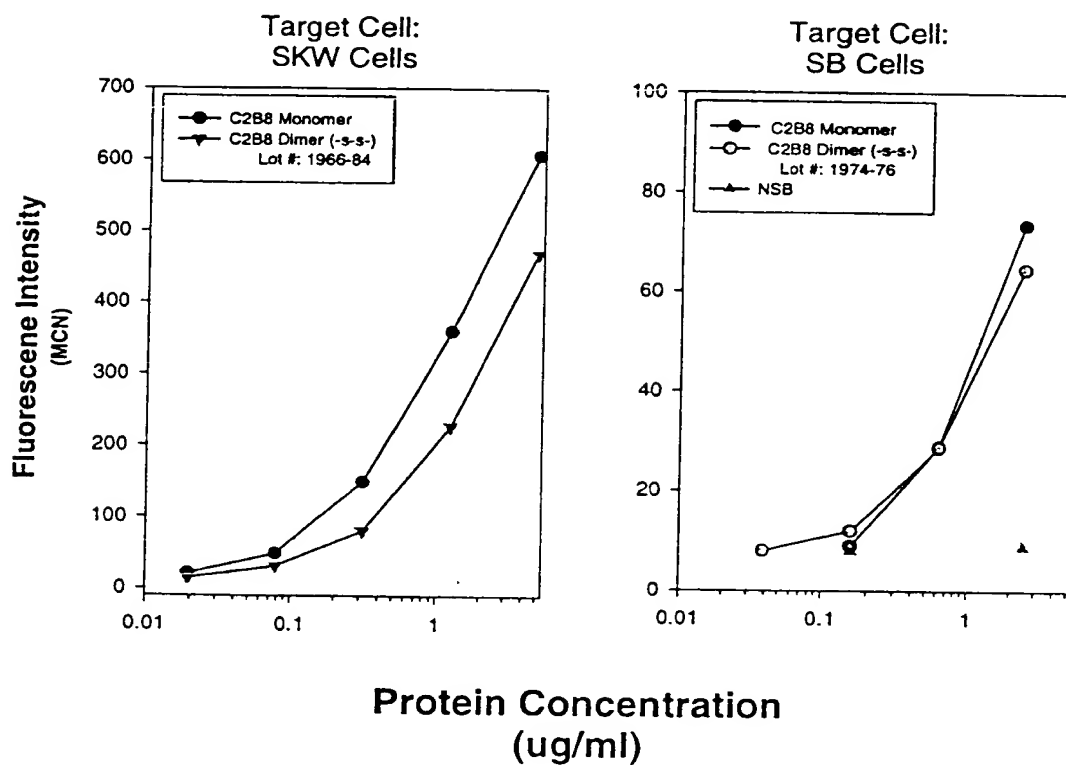


Fig 10: Competitive Binding Assay of C2B8 and C2B8 (-s-s-) Homodimer on SKW cells

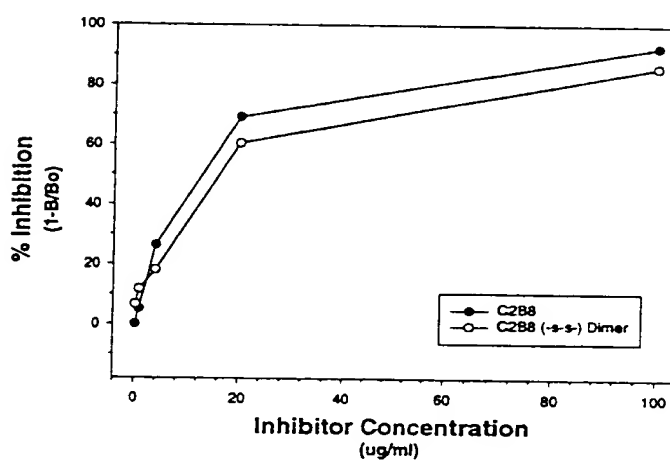


Fig. 11
Binding of α CD20/ α CD23 Heterodimer
(C2B8/p5E8, Lot #: 1966/84)
to SKW and DHL-4 Cell Lines

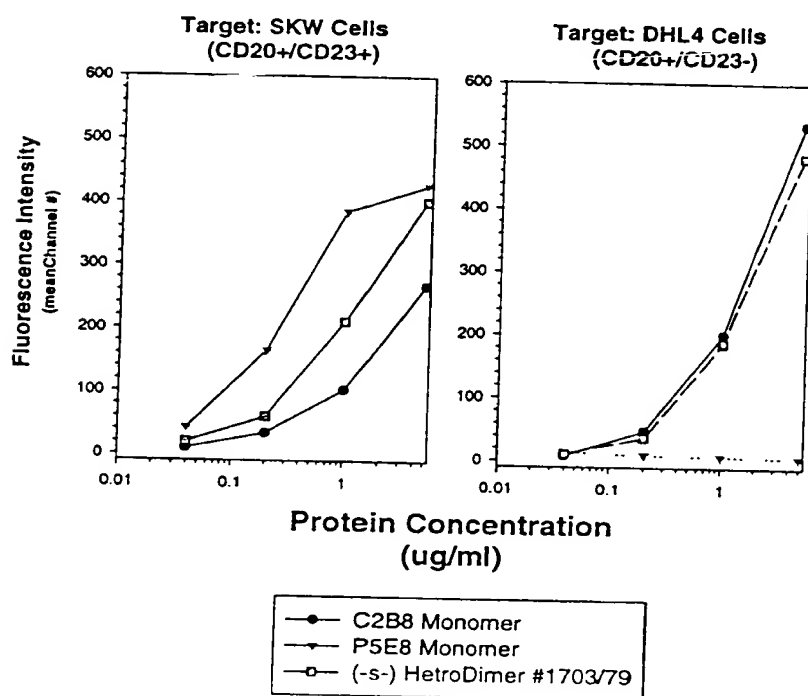


Fig: 12

Binding of aCD20 C2B8 Homodimer and
aCD20/aCD23 C2B8/p5E8 Heterodimer to
SKW cells (CD20+/CD23+)

Fig. XX: MAb binding to SKW cells (CD20+/CD23+).

SKW cells were incubated on ice with either PBS (filled bar) or murine (αCD20) Mab 2B8 (hatched bar) before staining with 10 μg/ml Mab C2B8, p5E8, C2B8 Homodimer and C2B8/p5E8 Heterodimer

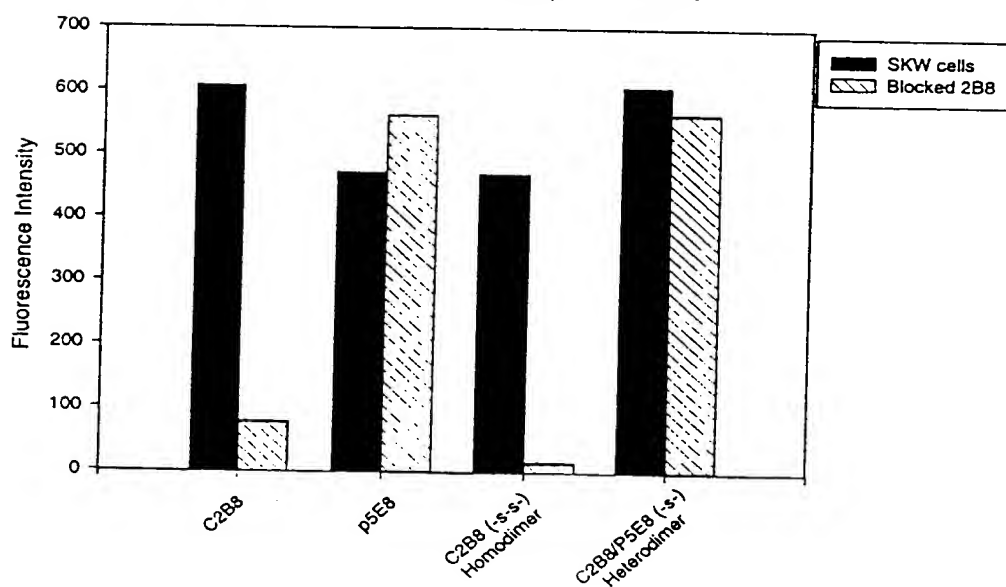


Fig. 13

s279: Antitumor Activity of C2B8 Chemical (-S-S-) Dimers on Daudi Tumor Xenografts

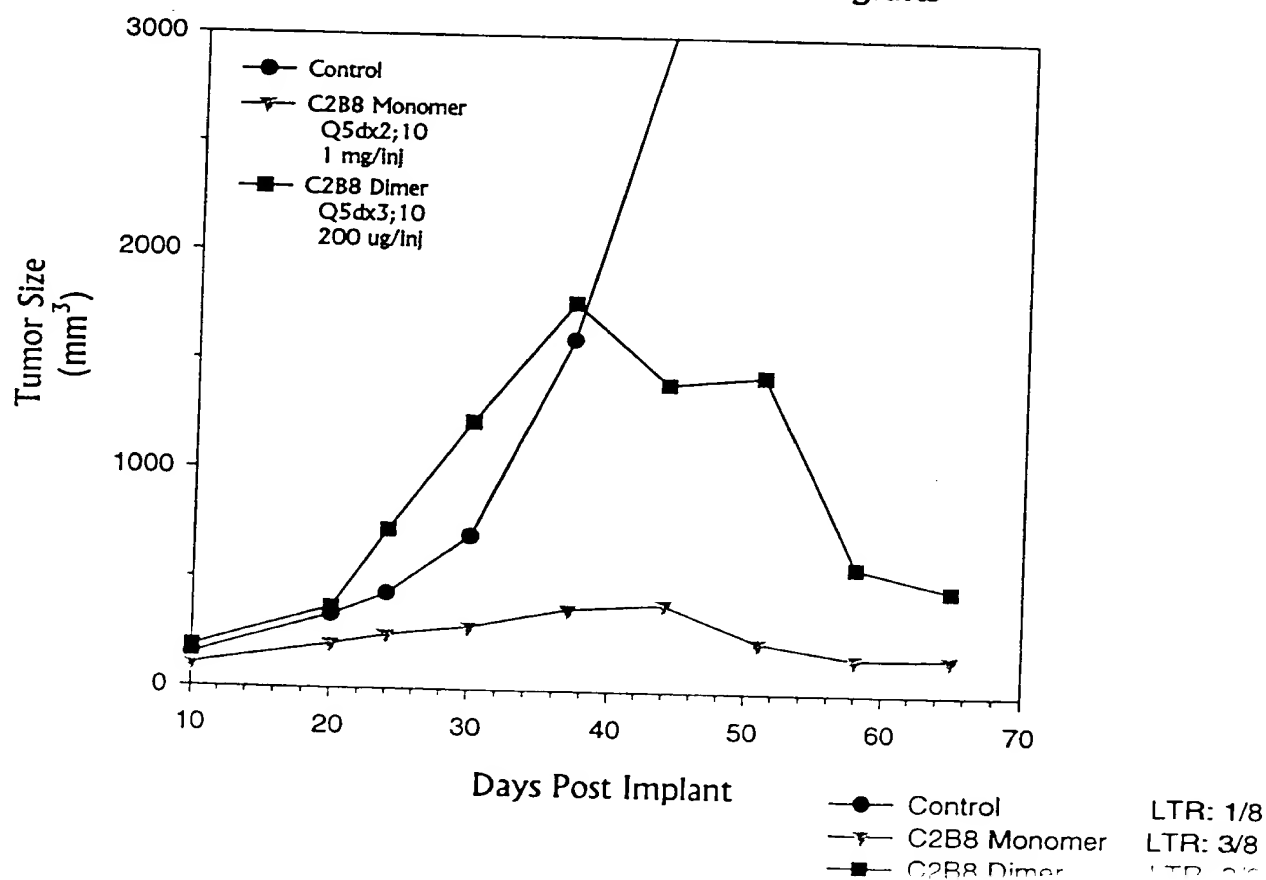


Fig. 14

286: Anti Tumor Activity of C2B8 (-s-s-) Dimers On Daudi Tumor Xenografts

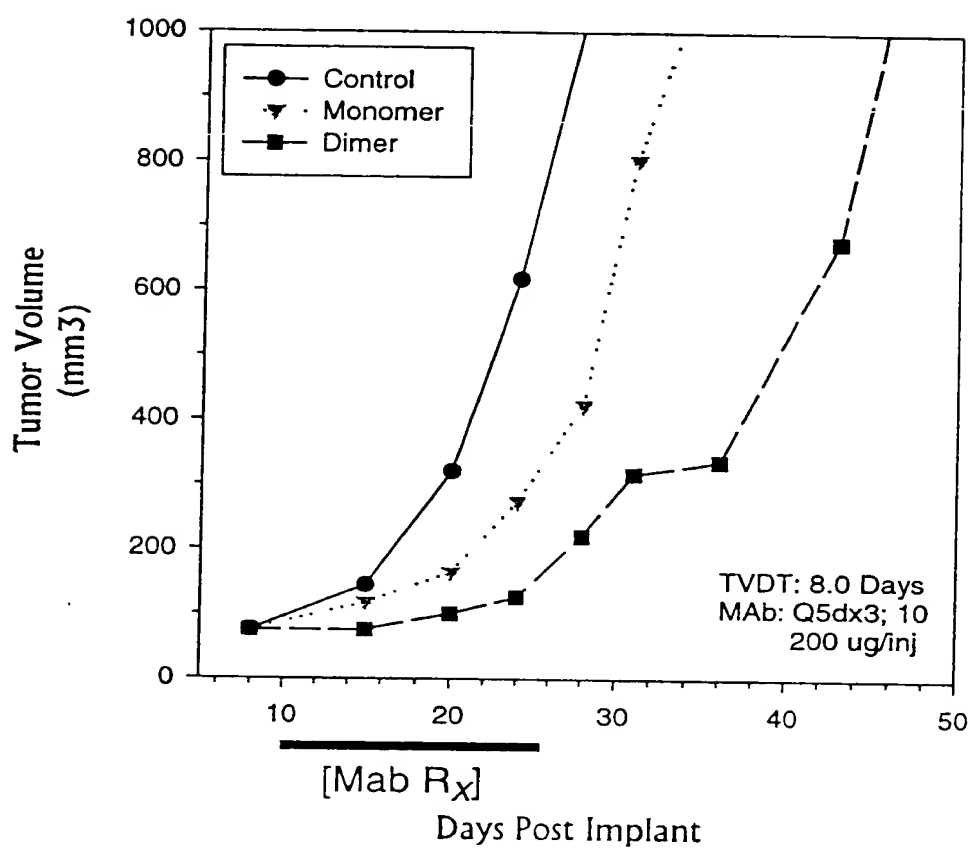


Fig. 15 **Apoptotic Activity of C2B8** **(-s-s-) Homodimer**

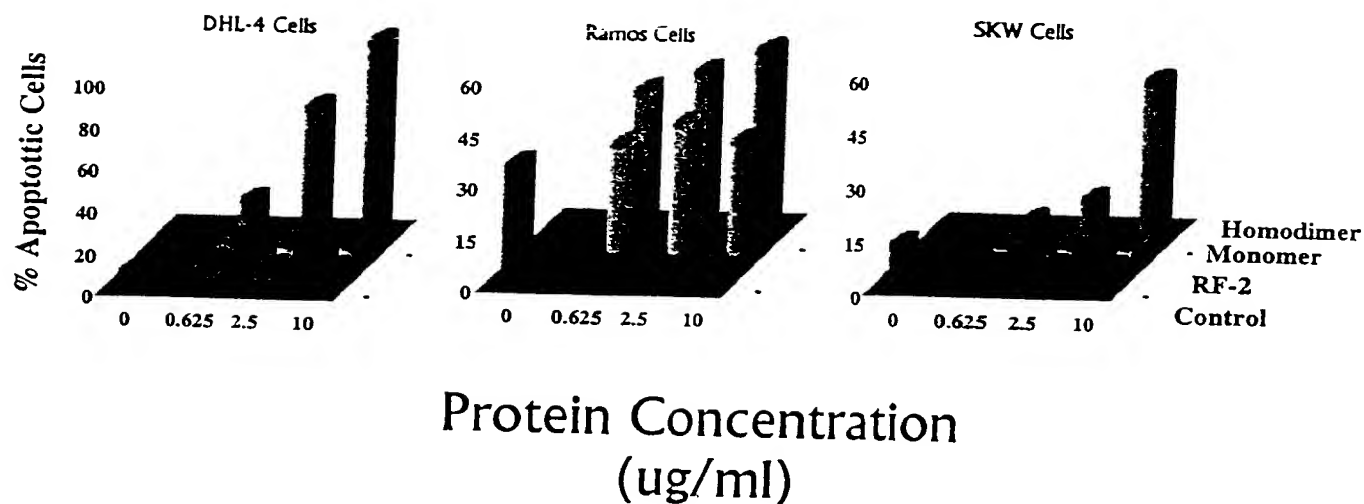


Fig 16
Apoptotic Activity of C2B8/p5E8
(-s-) Hetrodimer

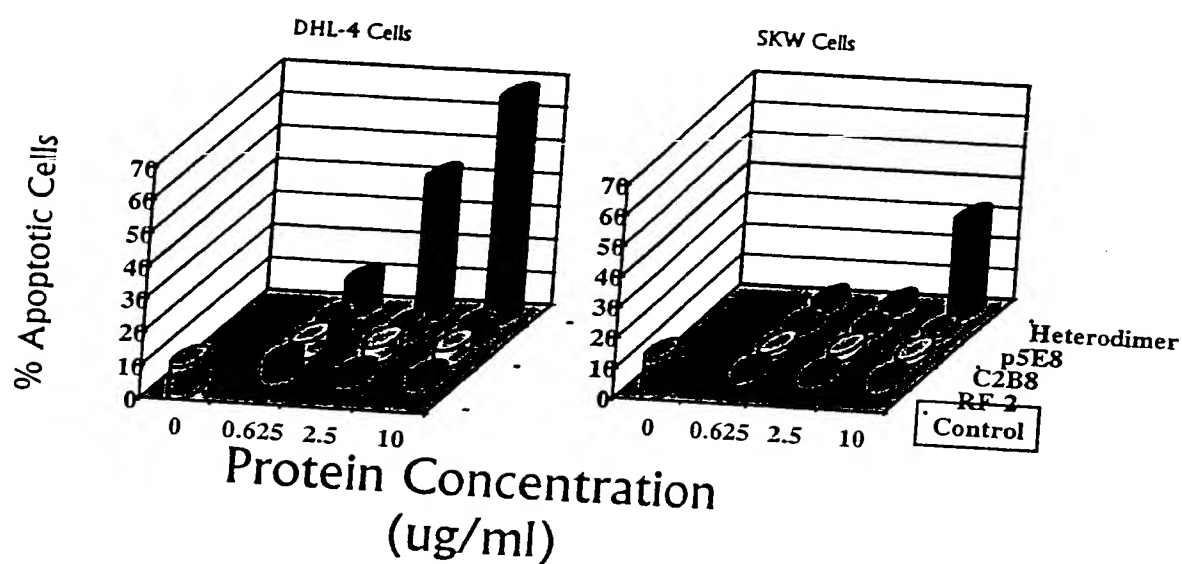


Fig. 17:
Growth Inhibition of B-Lymphoma CD20/CD23 Positive
Cell Lines (SB and SKW) after 96 hour Continuous
Exposure to MAb

